Remarks:

Claims

Reconsideration of the rejections is respectfully requested.

The number of total claims and of independent claims remains less than the amount for which fees were previously paid.

Claim 43 has been amended to correct a typographical error. No new matter is added.

Claim Rejection - 35 U.S.C. §102(b)

Claims 27, 29, 32, 34, 38, 43 and 44 stood rejected under 35 U.S.C. §102(b) based on an assertion that the claims are anticipated by Helminen et al. (J. Infec. Dis. 170; 867-872). The Examiner asserts that Helminen et al. discloses outer membrane proteins (OMPs) from M. catarrhalis cells. The Examiner alleges that the claims read on the disclosed OMPs in Helminen et al. In addition, the Examiner asserts that claim 61 reads on fusion proteins comprising OMPs and at least one other Moraxella antigen. In addition, the Examiner purports that Helminen et al. teaches OMPs in a buffer as immunogenic compositions. The Examiner contends that the burden is on Applicant to show a novel or unobvious difference between the claimed product and the product of the prior art.

Applicant respectfully disagrees. Applicant's invention is directed to BASB122 polypeptides and immunogenic fragments thereof. Applicant notes that a later published paper, Aebi et al. (Infect. Immun. 65, 4367-4377) concurrently submitted as Exhibit A discloses the deduced amino acid sequences for the UspA1 and UspA2 genes. The two disclosed proteins are said to be reactive to the monoclonal antibody disclosed in Helminen et al. The proteins have accession numbers AAB96359 (UspA1) and AAB96391 (UspA2), and PubMed Sequence Viewer printouts are submitted as Exhibit B. Applicant submits that the disclosed sequences have no significant similarity to the claimed SEQ ID NO:2.

Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. §102(b) is respectfully requested.

Information Disclosure Statement

Applicant has concurrently filed an Information Disclosure Statement (IDS) listing the references cited in the International Search Report for PCT/EP00/07365 on a PTO-1449 form. It

is noted that copies of the references have been received by the Office as indicated on Form PCT/DO/EO/903 (entitled, "Notification and Acceptance of Application under 35 U.S.C. 371 and 37 CFR 1.494 or 1.495"). It is respectfully requested that the listed references be included in the "References Cited" portion of any patent issuing from this application.

FEE DEFICIENCY

☒ If an extension of time is deemed required for consideration of this paper, please consider this paper to comprise a petition for such an extension of time; The Commissioner is hereby authorized to charge the fee for any such extension to Deposit Account No. 50-0258.

and/or

☒ If any additional fee is required for consideration of this paper, please charge Account No. 50-0258.

Closing Remarks

Applicant thanks the Examiner for the Office Action and believe this response to be a full and complete response to such Office Action. Accordingly, favorable reconsideration in view of this response and allowance of the pending claims are earnestly solicited.

Respectfully submitted,

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A Protective Epitope of *Moraxella catarrhalis* Is Encoded by Two Different Genes

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The high-molecular-weight UspA protein of *Moraxella catarrhalis* has been described as being both present on the surface of all *M. catarrhalis* disease isolates examined to date and a target for a monoclonal antibody (MAb 17C7) which enhanced pulmonary clearance of this organism in a mouse model system (M. E. Helminen et al., J. Infect. Dis. 170:867–872, 1994). A recombinant bacteriophage that formed plaques which bound MAb 17C7 was shown to contain a *M. catarrhalis* gene, designated *uspA1*, that encoded a protein with a calculated molecular weight of 88,271. Characterization of an isogenic *uspA1* mutant revealed that elimination of expression of UspA1 did not eliminate the reactivity of *M. catarrhalis* with MAb 17C7. In addition, N-terminal amino acid analysis of internal peptides derived from native UspA protein and Southern blot analysis of *M. catarrhalis* chromosomal DNA suggested the existence of a second UspA-like protein. A combination of epitope mapping and ligation-based PCR methods identified a second *M. catarrhalis* gene, designated *uspA2*, which also encoded the MAb 17C7-reactive epitope. The UspA2 protein had a calculated molecular weight of 62,483. Both the isogenic *uspA1* mutant and an isogenic *uspA2* mutant possessed the ability to express a very-high-molecular-weight antigen that bound MAb 17C7. Southern blot analysis indicated that disease isolates of *M. catarrhalis* likely possess both *uspA1* and *uspA2* genes. Both UspA1 and UspA2 most closely resembled adhesins produced by other bacterial pathogens.

The three most important causes of acute otitis media are *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae*, and *Moraxella catarrhalis*. *S. pneumoniae*, the most prevalent cause of middle ear disease, is responsible for at least 30% of these infections (15), whereas *M. catarrhalis* and nontypeable *H. influenzae* each account for approximately 15 to 26% of middle ear infections (5, 6, 36, 51, 57, 67). While the pathogenic potential of nontypeable *H. influenzae* strains has been recognized for some time (45), only recently has *M. catarrhalis* emerged as a significant cause of respiratory tract disease (6). Formerly named both *Branhamella catarrhalis* and *Neisseria catarrhalis*, this organism was long considered a nonpathogenic, commensal inhabitant of the upper respiratory tract (6). It is now accepted that *M. catarrhalis* is an important cause of otitis media in children. In fact, in a recent report, *M. catarrhalis* DNA could be detected by PCR in middle ear effusions from 46% of patients with chronic otitis media with effusion (51). *M. catarrhalis* also causes lower respiratory tract disease, including acute bronchitis and exacerbation of chronic bronchitis in adults, especially those with compromised respiratory function (23, 44, 49, 68).

The recent recognition of *M. catarrhalis* as an important pathogen in both the upper and lower respiratory tracts has resulted in increased interest in both its interactions with the human host (11–14, 17–19, 29, 35) and its antigenic composition. Outer membrane proteins constitute major antigenic determinants of this unencapsulated organism (3), and different strains share remarkably similar outer membrane protein profiles (3, 46). At least three different surface-exposed outer membrane antigens have been shown to be well conserved among *M. catarrhalis* strains; these include the 81-kDa CopB outer membrane protein (26), the heat-modifiable CD outer membrane protein (30, 47), and the very-high-molecular-weight UspA protein (27). Of these three antigens, both the CopB and UspA proteins have been shown to bind antibodies which exert biological activity (i.e., protection) against *M. catarrhalis* in an animal model (27, 47).

Previous studies revealed that at least one epitope of the UspA protein is exposed on the surface of all disease isolates of *M. catarrhalis* tested to date; this epitope is defined by its reactivity with the protective monoclonal antibody (MAb) 17C7 (27). The UspA protein of *M. catarrhalis* 035E migrates with an apparent molecular weight of at least 250,000, and the UspA proteins of other *M. catarrhalis* strains appear to be even larger (27, 34). In the present study, a *M. catarrhalis* strain 035E gene encoding a MAb 17C7-reactive protein was shown to encode an 88-kDa protein, designated UspA1, that contained a number of amino acid repeat motifs. Mutant analysis revealed that inactivation of the expression of the *uspA1* gene did not eliminate the reactivity of *M. catarrhalis* 035E with MAb 17C7. A combination of epitope mapping and PCR technology was used to identify a second *M. catarrhalis* gene encoding a 62-kDa protein, designated UspA2, which also contained the MAb 17C7-reactive epitope.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. catarrhalis* 035E, TTA24, and P44 have been described previously (26, 27, 65). *M. catarrhalis* TTA1 and TTA37 were obtained from transtracheal aspirates and provided by Steven Berk, East Tennessee State University, Johnson City. *M. catarrhalis* 25240 was obtained from the American Type Culture Collection, Rockville, Md. *M. catarrhalis* strains were routinely cultured at 37°C in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) or on BHI agar plates in an atmosphere of 95% air–5% CO_2. When appropriate, kanamycin was added to the BHI medium to a final concentration of 20 μg/ml. *Escherichia coli* DH5α, LE392, and XL1-Blue MRF′ (Stratagene, La Jolla, Calif.) were grown on Luria-Bertani medium (41) at

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TABLE 1. Bacteriophages and plasmids used in this study

Bacteriophage or plasmid	Description	Source
Bacteriophages		
LambdaGEM-11	Cloning vector	Promega Corp.
MEH200	LambdaGEM-11 containing an 11-kb insert of *M. catarrhalis* 035E DNA encoding the UspA1 protein	27
ZAP Express	Cloning vector	Stratagene
USP100	ZAP Express with a 2.7-kb fragment of DNA (containing *uspA1*) amplified from the chromosome of *M. catarrhalis* 035E	This study
USP200	ZAP Express with a 2.1-kb fragment of DNA (containing *uspA2*) amplified from the chromosome of *M. catarrhalis* 035E	This study
Plasmids		
pBS	Cloning vector, Amp^r	Stratagene
pJL501.6	pBS containing the 1.6-kb *Bgl*II-*Eco*RI fragment from MEH200	This study
pJL500.5	pBS containing the 600-bp *Bgl*II fragment from MEH200	This study
pUSPA1	pBS containing the partial *uspA1* ORF (bp 339 to 2984)	This study
pUSPA1KAN	pUSPA1 with a *kan* cartridge inserted into the partial *uspA1* ORF	This study
pGEX-4T-2	GST fusion protein vector	Pharmacia
pMF-3	pGEX-4T-2 containing bp 1185 to 1742 from *uspA1*	This study
pMF-4	pGEX-4T-2 containing bp 1638 to 2303 from *uspA1*	This study
pMF-4-1	pGEX-4T-2 containing bp 1722 to 1954 from *uspA1*	This study
pMF-4-2	pGEX-4T-2 containing bp 1934 to 2303 from *uspA1*	This study
pUSPA2	pBS containing the partial *uspA2* ORF (bp 649 to 2596)	This study
pUSPA2KAN	pUSPA2 with a *kan* cartridge inserted into the partial *uspA2* ORF	This study

37°C, supplemented with maltose (0.2%, wt/vol), 10 mM $MgSO_4$, and antimicrobial agents as necessary.

MAbs and immunological methods. MAb 17C7 is a murine immunoglobulin G (IgG) antibody reactive with the UspA protein of all *M. catarrhalis* disease isolates tested to date (27). This MAb was used in the form of hybridoma culture supernatant fluid in all experiments. The colony blot-radioimmunoassay has been described before (22).

Cloning vectors. Plasmid and bacteriophage cloning vectors utilized in this study and the recombinant derivatives of these vectors are listed in Table 1. MEH200, the original recombinant bacteriophage clone that produced plaques reactive with the UspA-specific MAb 17C7, has been described previously (27).

Genetic techniques. Standard recombinant DNA techniques, including plasmid isolation, restriction enzyme digestions, DNA modifications, ligation reactions, and transformation of *E. coli*, were performed as described previously (41, 53). The use of electroporation to construct isogenic mutants of *M. catarrhalis* has been described (28); the 1.2-kb *kan* cartridge used for these experiments was excised from pUC4K (Pharmacia-LKB Biotechnology, Piscataway, N.J.) by digestion with *Bam*HI.

Southern blot analysis. Southern blot analysis of chromosomal DNA fragments derived from *M. catarrhalis* strains was performed as described before (26). Oligonucleotide probes were labeled with a 3'-end labeling fluorescein kit (Dupont NEN, Wilmington, Dela.). Double-stranded DNA probes were labeled with ^{32}P with a random-primed DNA labeling kit (Boehringer-Mannheim, Indianapolis, Ind.).

PCR. The PCR was performed with the GeneAmp kit (Perkin-Elmer, Branchburg, N.J.). All reactions were carried out as described in the manufacturer's instructions. To amplify products from total genomic DNA, 1 μg of *M. catarrhalis* chromosomal DNA and 100 ng of each primer were used in each 100-μl reaction mixture. Ligation-based PCR experiments (i.e., vector-anchored PCR) (20, 37) were performed essentially as described before (33), except as noted. Oligonucleotide primers used in this study were designated P1 to P18 in Fig. 2 and 6.

Nucleotide sequence analysis. Nucleotide sequence analysis of DNA fragments in recombinant plasmids or derived from PCR was performed with an Applied Biosystems (Foster City, Calif.) model 373A automated DNA sequencer. Nucleotide sequence analysis of a *M. catarrhalis* DNA insert in a bacteriophage was facilitated by use of a presequencing kit for linear double-stranded DNA (United States Biochemicals, Cleveland, Ohio); this DNA was analyzed by double-stranded sequencing methods (70). Nucleotide sequence information was analyzed with the Intelligenetics suite package and programs from the University of Wisconsin Genetics Computer Group sequence analysis package (version 8.1) (10). Analysis of protein hydrophilicity by the method of Kyte and Doolittle (38) and analysis of repeated amino acid sequences within proteins were performed with the MacVector 6.0 software protein matrix analysis package (Oxford Molecular Ltd., Campbell, Calif.). The GAP alignment algorithm contained in the University of Wisconsin software package was used for direct comparison of the amino acid sequences of entire proteins. The ClustalW program for pairwise alignment with the Blosum 30 scoring matrix, as contained in MacVector 6.0, was utilized to determine similarity between peptides.

Identification of recombinant bacteriophage. Lysates were generated from *Escherichia coli* cells infected with recombinant bacteriophage by use of the plate lysis method as described previously (27). MAb-based screening of plaques formed by recombinant ZAP Express bacteriophage on *E. coli* XL1-Blue MRF' cells was performed as described in the manufacturer's instructions. Briefly, nitrocellulose filters soaked in 10 mM IPTG (isopropyl-β-D-thiogalactopyranoside) were applied to the surface of agar plates 5 h after bacteriophage infection of the bacterial lawn. After overnight incubation at 37°C, the nitrocellulose pads were removed, washed with phosphate-buffered saline (PBS) containing 0.5% (vol/vol) Nonidet P-40 and 5% (wt/vol) skim milk (PBS-N), and incubated with hybridoma culture supernatant containing MAb 17C7 for 4 h at room temperature. After four washes with PBS-N, PBS-N containing ^{125}I-labeled goat anti-mouse IgG was applied to each pad. After overnight incubation at 4°C, the pads were washed four times with PBS-N, blotted dry, and exposed to film.

Characterization of *M. catarrhalis* protein antigens. Outer membrane vesicles were extracted from BHI broth-grown *M. catarrhalis* cells by the EDTA-buffer method (48). Proteins present in these vesicles were solubilized by heating at 100°C for 5 min in digestion buffer (21) and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5% (wt/vol) polyacrylamide separating gels. These SDS-PAGE-resolved proteins were transferred electrophoretically to nitrocellulose, and Western blot (immunoblot) analysis was performed as described previously with MAb 17C7 as the primary antibody (32). Radioiodinated goat anti-mouse IgG was used as the secondary antibody.

N-terminal amino acid sequence analysis. Proteins present in outer membrane vesicles or cell envelopes (24) prepared from *M. catarrhalis* 035E were resolved by SDS-PAGE, and the UspA protein band was excised. This protein was electroeluted and then again subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane by the method of Matsudaira (42). The protein was then digested with trypsin, endoproteinase Lys-C (Promega), or cyanogen bromide. The resultant peptides were resolved by high-performance liquid chromatography and subjected to N-terminal amino acid sequence analysis as described before (25).

Construction and analysis of fusion proteins. A glutathione *S*-transferase (GST) fusion protein system was used for localization of the epitope in UspA1 that bound MAb 17C7. Pairs of oligonucleotide primers were designed to amplify 400- to 600-bp fragments spanning the *uspA1* gene from *M. catarrhalis* 035E. Each of these primers had either a *Bam*HI site or a *Xho*I site at the 5' end, thereby allowing directional in-frame cloning of the amplified product into the *Bam*HI- and *Xho*I-digested pGEX-4T-2 vector (Pharmacia). Each of the resultant plasmid constructs was confirmed by nucleotide sequence analysis. Whole-cell lysates prepared from each recombinant *E. coli* strain were probed in Western blot analysis independently with MAb 17C7 and with a polyclonal mouse antiserum specific for GST (to verify expression of the fusion protein). Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin was used as the secondary antibody.

Nucleotide sequence accession number. The complete nucleotide sequences of the *uspA1* and *uspA2* genes from *M. catarrhalis* 035E have been deposited in GenBank under accession numbers U57551 and U86135, respectively.

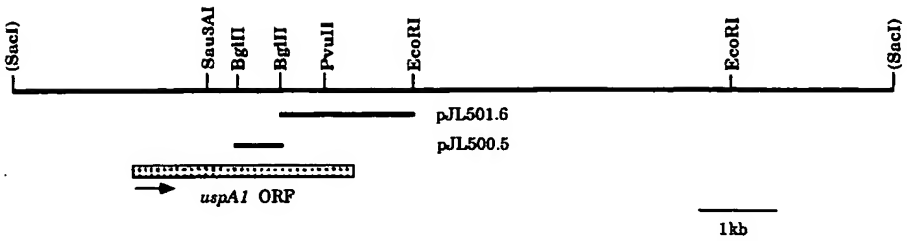


FIG. 1. Partial restriction enzyme map of the *M. catarrhalis* DNA insert in MEH200 and its derivatives. Relevant restriction sites are indicated; the *Sau*3AI site is not the only *Sau*3AI site in the insert. The 1.6-kb internal *Bgl*II-*Eco*RI fragment and the 600-bp internal *Bgl*II fragment were subcloned into pBS, creating the plasmids pJL501.6 and pJL500.5, respectively. The inserts in these plasmids are indicated by black bars. The shaded box indicates the position of the 2.5-kb *uspA1* ORF; the arrow beneath the left corner of this ORF indicates the direction of transcription. The use of parentheses around the *Sac*I sites indicates that these sites are in the vector DNA.

RESULTS

Subcloning and analysis of the *uspA1* gene. An 11-kb fragment of chromosomal DNA from *M. catarrhalis* 035E encoding a MAb 17C7-reactive protein was originally obtained from a bacteriophage-based genomic library (27). For the purpose of clarity, the *M. catarrhalis* gene encoding this MAb 17C7-reactive protein has now been designated *uspA1*. Efforts to subclone the entire 11-kb *M. catarrhalis* DNA insert from the recombinant bacteriophage MEH200 (Fig. 1) into a plasmid vector were unsuccessful, even when very-low-copy-number vectors (e.g., pLG338 [62]) were utilized for this purpose. When attempts were made to introduce various restriction fragments from this 11-kb insert into plasmid vectors, four fragments comprising 8.2 kb of contiguous DNA were subcloned successfully. These included the 600-bp *Bgl*II fragment, the 1.6-kb *Bgl*II-*Eco*RI fragment, the 4-kb *Eco*RI fragment, and the 2-kb *Eco*RI-*Sac*I fragment (Fig. 1). However, none of these recombinant clones expressed the MAb 17C7-reactive antigen.

Preliminary nucleotide sequence analysis of the 4-kb *Eco*RI and the 2-kb *Eco*RI-*Sac*I fragments revealed that these represented chimeras containing both *M. catarrhalis* DNA and *E. coli* DNA; no further analyses of these fragments were performed. In contrast, nucleotide sequence analysis of the 600-bp *Bgl*II fragment in the recombinant plasmid pJL500.5 and 1.6-kb *Bgl*II-*Eco*RI fragment in the recombinant plasmid pJL501.6 (Fig. 1) revealed the presence of a partial open reading frame (ORF) that encoded an incomplete 54-kDa polypeptide. The beginning of this partial ORF was inferred to be localized within the 2.8-kb *Sac*II-*Bgl*II fragment (Fig. 1).

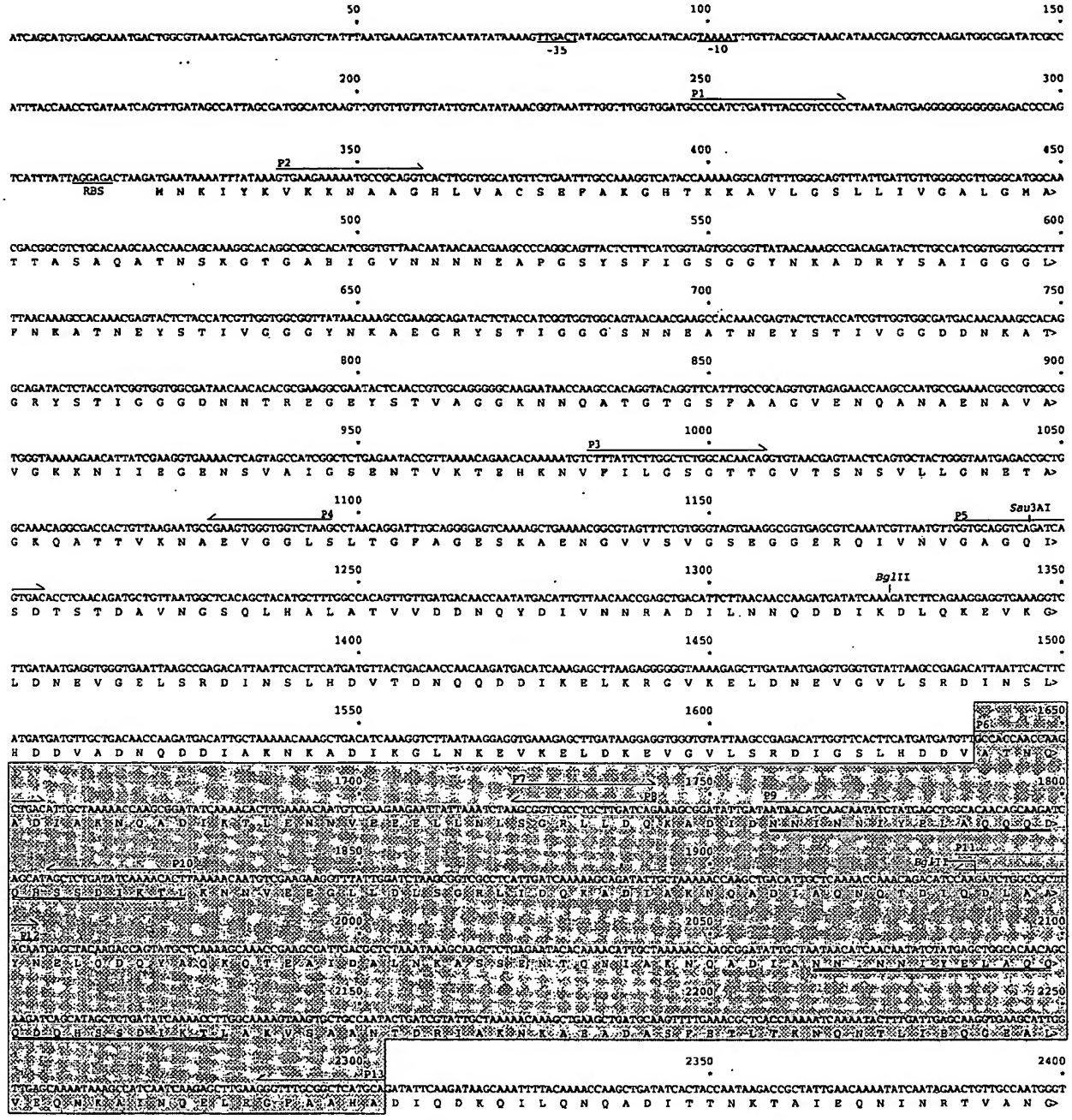
All attempts to subclone the 2.8-kb *Sac*I-*Bgl*II fragment were unsuccessful. However, double-stranded sequencing of the bacteriophage DNA, although very inefficient, yielded approximately 500 nucleotides of additional sequence 5′ from the first *Bgl*II site in MEH200; this new sequence included a *Sau*3AI site (Fig. 1). A 26-nucleotide probe specific for a region 5′ from this *Sau*3AI site in the MEH200 insert (see P3 in Fig. 2) was shown to hybridize to a 1.2-kb *Sau*3AI fragment from *M. catarrhalis* 035E by Southern blot analysis (data not shown). This allowed us to infer that this 1.2-kb *Sau*3AI fragment contained the 5′ end of the putative *uspA1* ORF.

Use of ligation-based PCR for nucleotide sequence analysis. *Sau*3AI-digested chromosomal DNA fragments from this strain were ligated into the *Bam*HI site in pBluescript II SK+ (pBS), and the ligation reaction mixture was precipitated, dried, and resuspended in 50 μl of sterile distilled water. This material was subjected to PCR amplification with an oligonucleotide primer specific for a region immediately 5′ from the relevant *Sau*3AI site in MEH200 (see P4 in Fig. 2) and a primer specific for the pBS T7 promoter. After PCR amplification, the entire reaction mixture was subjected to agarose gel electrophoresis. A 1.2-kb band (corresponding to the predicted size of the desired *Sau*3AI fragment) was obtained and used as the template in a second round of PCR amplification with the same primers.

Nucleotide sequence analysis of this final PCR product revealed that it contained the likely translational start site, together with a putative promoter region, to complete the partial ORF encoded by the inserts in the recombinant plasmids pJL501.6 and pJL500.5 (Fig. 1). The position of this ORF in the original *M. catarrhalis* DNA insert in MEH200 is shown in Fig. 1. Primers P1 and P14 (see Fig. 2) were used to amplify a 2.7-kb fragment containing the entire *uspA1* ORF directly from the chromosome of *M. catarrhalis* 035E, and both strands of this 2.7-kb PCR product were sequenced in their entirety to confirm the nucleotide sequence information derived from the ligation-based PCR product.

Features of the *uspA1* gene and its encoded protein product. The nucleotide sequence of the *M. catarrhalis* 035E *uspA1* gene and the deduced amino acid sequence of the UspA1 protein are shown in Fig. 2. The ORF, containing 2,496 nucleotides, encoded a protein product of 831 amino acids, with a calculated molecular weight of 88,271. The likely translational start site was located at nucleotide 321 (Fig. 2); this ATG start codon is located 7 nucleotides downstream from a sequence (5′-AGGA-3′) with homology to ribosome binding sites (56). Putative −10 and −35 consensus sequences were also identified upstream from the putative start codon (Fig. 2). A possible stem-loop terminator sequence was located between nucleotides 2841 and 2874. There were no ORFs located within 300 bp 5′ from the start of the *uspA1* gene. On the opposite DNA strand, approximately 200 bp from the 3′ end of the *uspA1* gene, there was an ORF encoding a predicted product similar to the *E. coli* P14 protein (52). The predicted protein product of the *uspA1* ORF was fairly hydrophilic and was distinguished by its high content of a number of different amino acid repeat motifs containing at least three predicted leucine zippers (data not shown). The significance of these motifs remains to be determined.

Similarity of UspA1 to other proteins. When the nucleotide sequence of *uspA1* was analyzed through the National Center for Biotechnology Information by use of the BLAST network service to search GenBank (2, 16), the *hsf* gene product of *H. influenzae* type b (61) was found to be the prokaryotic protein most similar to this *M. catarrhalis* antigen. This *H. influenzae* protein forms short, thin fibrils on the surface of *H. influenzae* type b that promote attachment to human epithelial cells (60). Other proteins retrieved from database searches as having



GAATGGACA

FIG. 2. Nucleotide sequence of the *uspA1* gene from *M. catarrhalis* 035E together with the deduced amino acid sequence. Putative −35 and −10 regions are indicated; a possible ribosome binding site (RBS) is also indicated. An inverted repeat located 3′ from the ORF is indicated by opposing arrows at nucleotides 2841 to 2874. Oligonucleotide primers (P1 to P14) used for PCR amplification are indicated by arrows placed above the relevant sequences. The shaded box contains the nucleotide sequence encoding the 222 amino acids present in the MF-4 fusion protein. The 23-residue peptides common to both the MF-4-1 and MF-4-2 fusion proteins are double underlined in this shaded box. Relevant restriction sites are indicated.

some similarity with UspA1 included myosin heavy chains from a number of species.

N-terminal amino acid sequence analysis of internal UspA peptides. To confirm the deduced amino acid sequence of the UspA1 protein, we performed N-terminal amino acid sequence analysis on peptide fragments from native UspA protein. The very-high-molecular-weight UspA protein (i.e., apparent molecular weight of greater than 250,000 in SDS-PAGE [27]) from *M. catarrhalis* 035E was resolved by SDS-PAGE, electroeluted, and digested with various proteinases or with cyanogen bromide. When the resultant peptides were subjected to N-terminal amino acid sequence analysis, several of the peptides exactly or closely matched peptides located near the center of the deduced amino acid sequence of the UspA1 protein (Table 2). However, four additional peptides with sequences that had only weak similarity to or were not present in the deduced amino acid sequence were found (Table 2). The degree of identity between the sequences of these four peptides and the deduced amino acid sequence of the UspA1 protein ranged from 26 to 33% (ClustalW score range, 35 to 43). These findings first raised the possibility that there might be a second protein, similar to UspA1, present in the electroeluted UspA protein band.

Southern blot analysis with a *uspA1* gene probe. To obtain preliminary genetic evidence for the possible existence of a second gene encoding a UspA1-like protein, we used a DNA fragment from the *uspA1* gene to probe chromosomal DNA from several *M. catarrhalis* strains. A 600-bp *Bgl*II-*Pvu*II fragment from pJL501.6, containing the 3′ end of the *uspA1* gene from strain 035E (Fig. 1), was used to probe a *Pvu*II digest of chromosomal DNA from strain 035E and five additional strains of *M. catarrhalis*. Interestingly, each strain yielded two *Pvu*II fragments that hybridized with this probe (Fig. 3, lanes A to F). This finding reinforced the possibility that there was another *uspA1*-like gene in the *M. catarrhalis* chromosome.

Construction and characterization of an isogenic *uspA1* mutant. Mutant analysis was utilized to determine conclusively whether there were two similar UspA proteins expressed by *M. catarrhalis*. Oligonucleotide primers (P2 and P14 [Fig. 2], each with a *Bam*HI site at the 5′ end) were used to amplify a 2.6-kb product containing most of the *uspA1* ORF except the first 18 nucleotides; this PCR product was cloned into the *Bam*HI site of pBS, yielding the recombinant plasmid pUSPA1 (Table 1). A 0.6-kb *Bgl*II fragment from the middle of this cloned fragment was excised and replaced by a *Bam*HI-ended *kan* cartridge. This new plasmid, designated pUSPA1KAN, was linearized by digestion with *Eco*RI and used to electroporate the wild-type *M. catarrhalis* strain 035E as described before (28). Approximately 5,000 kanamycin-resistant transformants were obtained; several picked at random were found to be still reactive with MAb 17C7 in the colony blot-radioimmunoassay. One of these kanamycin-resistant strains, designated *M. catarrhalis* 035E.1, was randomly chosen for further testing.

Southern blot analysis confirmed that 035E.1 was an isogenic *uspA1* mutant. When chromosomal DNA from both the wild-type parent strain and strain 035E.1 was digested with *Pvu*II and probed in Southern blot analysis with the 600-bp *Bgl*II-*Pvu*II fragment from pJL501.6, the wild-type strain (Fig. 3, lane A) exhibited 2.6- and 2.8-kb *Pvu*II fragments which bound this *uspA1*-derived probe. In contrast, the mutant strain (Fig. 3, lane G) had 2.6- and 3.4-kb *Pvu*II fragments that bound this probe. The presence of the 3.4-kb *Pvu*II fragment was the result of allelic exchange involving the mutated *uspA1* gene containing the *kan* cartridge; only the 3.4-kb *Pvu*II fragment of the mutant bound a *kan* cartridge probe (data not shown). Therefore, we inferred from this result that the 2.8-kb *Pvu*II fragment from strain 035E (Fig. 3, lane A) contained the *uspA1* ORF.

TABLE 2. Amino acid sequence of peptides derived from the SDS-PAGE-purified, very-high-molecular-weight UspA protein from *M. catarrhalis* 035E

Type of peptides	Sequences
Similar or identical[a]	KALESNVEEGLLDLSGR
	ALESNVEEGLLELSGRTIDQR
	NQAHIANNINXIYELAQQQDQK[c]
	NQADIAQNQTDIQDLAAYNELQ
	ATHDYNERQTEA
	KASSENTQNIAK
Other[b]	MILGDTAIVSNSQDNKTQLKFYK
	AGDTIIPLDDDXXP
	LLHEQQLXGK
	IFFNXG

[a] Peptides identical or very similar to peptides contained in the deduced amino acid sequence of the UspA1 protein.

[b] Peptides which match poorly or not at all with the deduced amino acid sequence of the UspA1 protein.

[c] X indicates a residue that was not identified.

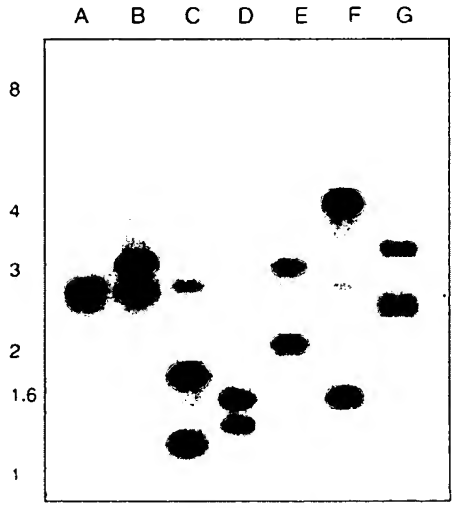


FIG. 3. Southern blot analysis of *Pvu*II-digested chromosomal DNA from wild-type and mutant strains of *M. catarrhalis* with a probe containing *uspA1* DNA. The 600-bp *Bgl*II-*Pvu*II fragment from pJL501.6 was used as the probe. Lanes: A, 035E; B, TTA24; C, TTA1; D, TTA37; E, P44; F, ATCC 25240; G, *uspA1* mutant strain 035E.1. Kilobase position markers are indicated to the left of the figure.

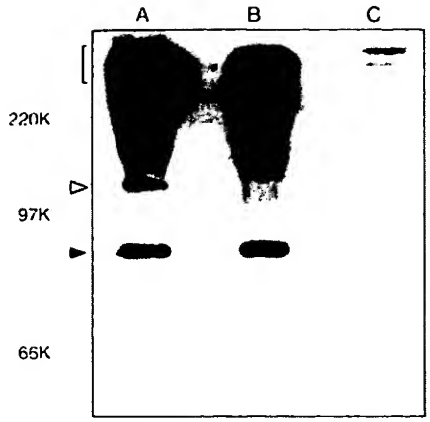


FIG. 4. Western blot analysis of outer membrane proteins in wild-type and mutant *M. catarrhalis* strains by use of autoradiography. Proteins present in EDTA-extracted outer membrane vesicles from the wild-type strain 035E (lane A), the isogenic *uspA1* mutant strain 035E.1 (lane B), and the isogenic *uspA2* mutant strain 035E.2 (lane C) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with MAb 17C7 followed by radioiodinated goat anti-mouse immunoglobulin. The bracket on the left indicates the very-high-molecular-weight form of the UspA protein. The open arrowhead indicates the 120-kDa, putative monomeric form of the UspA1 protein. The closed arrowhead indicates the 85-kDa, putative monomeric form of the UspA2 protein. This autoradiogram had to be overexposed to detect the 120- and 85-kDa proteins; shorter exposure times revealed that the very-high-molecular-weight form of the UspA protein expressed by both the wild-type strain and the *uspA1* mutant formed a discrete band that bound MAb 17C7 and also gave rise to a number of apparent degradation products which migrated slightly faster in SDS-PAGE. Molecular weight position markers (K, thousand) are shown to the left of the figure.

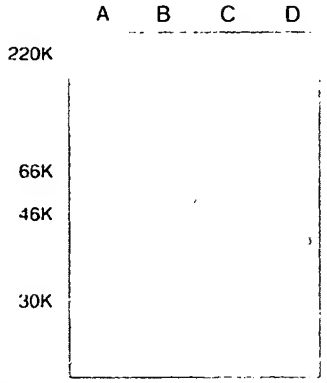


FIG. 5. Western blot analysis of the reactivity of UspA1-derived fusion proteins with MAb 17C7. The MAb 17C7-reactive fusion proteins MF-4 (lane B), MF-4-1 (lane C), and MF-4-2 (lane D) were produced by plasmid constructs generated as described in Results. Fusion protein MF-3 (lane A) contains amino acid residues 289 to 474 from UspA1; the plasmid construct expressing this fusion protein was produced by use of oligonucleotide primers P5 and P8 in Fig. 2 for PCR; it is included here as a negative control. Molecular weight position markers (K, thousand) are shown to the left of the figure.

Identification of MAb 17C7-reactive proteins. Western blot analysis of outer membrane vesicles of the *uspA1* mutant strain 035E.1 (Fig. 4, lane B) revealed that this mutant still expressed the very-high-molecular-weight protein reactive with MAb 17C7 and at a level equivalent to that expressed by the wild-type strain (Fig. 4, lane A). However, long-term exposure of this autoradiogram revealed that the *uspA1* mutant (Fig. 4, lane B) lacked expression of an antigen with an apparent molecular weight of approximately 120,000 that was expressed by the wild-type strain (Fig. 4, lane A).

The fact that this *uspA1* mutant still expressed the very-high-molecular-weight UspA antigen reactive with MAb 17C7 indicated that there had to be a second gene in *M. catarrhalis* 035E that encoded a MAb 17C7-reactive protein. In this context, it should be noted that both the wild-type strain and the *uspA1* mutant (Fig. 4, lanes A and B, respectively) expressed an antigen with an apparent molecular weight of approximately 85,000 that bound MAb 17C7. Identification of the gene encoding this 85-kDa protein was successfully accomplished by use of a combination of epitope mapping and PCR methods as described below.

Localization of the UspA1 epitope that binds MAb 17C7. The UspA1 epitope which bound MAb 17C7 was localized by use of the nucleotide sequence of the *uspA1* gene to construct GST-fusion proteins. The epitope that bound MAb 17C7 was localized first to a 222-amino-acid span (encoded by nucleotides 1638 to 2303 [Fig. 2]) contained in the fusion protein MF-4 (Fig. 5, lane B). The oligonucleotide primers (P6 and P13) used in the PCR to amplify the relevant nucleotide sequence from *M. catarrhalis* 035E chromosomal DNA are depicted in Fig. 2. Further analysis of the UspA1-derived amino acid sequence in the MF-4 fusion construct involved the production of fusion proteins containing 78 amino acid residues (MF-4-1, derived from the use of primers P7 and P12 [Fig. 2]) and 123 amino acid residues (MF-4-2, derived from the use of primers P11 and P13). Both MF-4-1 and MF-4-2 bound MAb 17C7 (Fig. 5, lanes C and D, respectively) and had in common a 23-residue region, suggesting, although not proving, that this 23-residue region contained the epitope that bound MAb 17C7.

Identification of a second gene in *M. catarrhalis* 035E encoding the MAb 17C7-reactive epitope. It is important to note that the nucleotide sequence encoding the 23-amino-acid region common to both MF-4-1 and MF-4-2 was present in the 600-bp *Bgl*II-*Pvu*II fragment (Fig. 2) used in the Southern blot analysis described above (Fig. 3). This finding suggested that the epitope that bound MAb 17C7 might be encoded by DNA present in both the 2.6- and 2.8-kb *Pvu*II fragments from *M. catarrhalis* 035E that hybridized with this probe (Fig. 3, lane A). Moreover, by comparison with the Southern blot results obtained with the isogenic *uspA1* mutant (Fig. 3, lane G), it was apparent that the wild-type 2.8-kb *Pvu*II fragment (Fig. 3, lane A) contained *uspA1* DNA and that the wild-type 2.6-kb *Pvu*II fragment (Fig. 3, lane A) likely represented all or part of another gene encoding this same epitope.

This hypothesis was tested by means of the previously described ligation-based PCR system. Chromosomal DNA from the isogenic *uspA1* mutant was digested to completion with *Pvu*II and resolved by agarose gel electrophoresis. Fragments ranging in size from 2 to 3 kb were excised from the agarose, blunt ended, and ligated into the *Eco*RV site in pBS. This ligation reaction mixture was precipitated and used in a PCR amplification. Each PCR contained an oligonucleotide primer for the vector together with an oligonucleotide primer (either P9 or P10 [Fig. 2]) derived from the DNA near the center of the MF-4 insert. This approach yielded a 1.7- to 1.8-kb product with the vector and P10 primers and a 0.8- to 0.9-kb product with the vector and P9 primers. It should be noted that the sum of the sizes of these two bands is approximately the same as the 2.6-kb size of the desired DNA fragment.

Nucleotide sequence analysis of these two PCR products revealed the presence of a partial ORF in each; one contained a putative translation initiation codon, and the other contained a termination codon. When joined at the region containing the P9 and P10 primers (Fig. 6), these two partial ORFs formed a

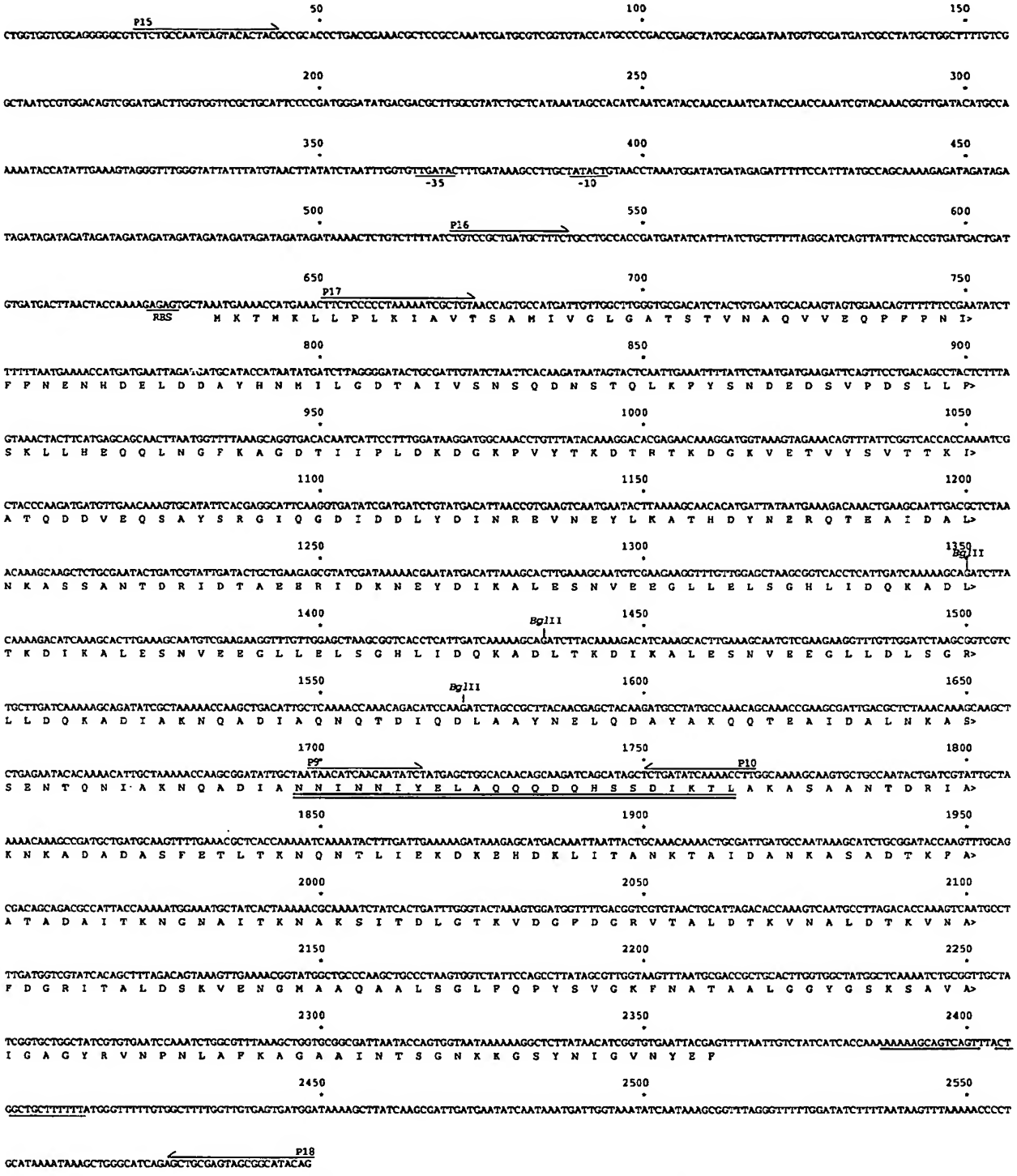


FIG. 6. Nucleotide sequence of the *uspA2* gene from *M. catarrhalis* 035E and the deduced amino acid sequence of the UspA2 protein. Putative −35, −10, and ribosome binding sites (RBS) are indicated. Fifteen repeats of the tetranucleotide AGAT are present between nucleotides 439 and 499. Opposing arrows indicate an inverted repeat immediately downstream from the ORF. Oligonucleotide primers (P15 to P18) used for PCR-based amplification of selected regions of this sequence from the chromosome of the wild-type *M. catarrhalis* strain 035E are indicated by arrows placed above the relevant sequences. Primers P9 and P10 are included here to indicate the *uspA2* DNA regions that bound these primers in the ligation-based PCR experiment. The *Bgl*II sites used for insertion of the *kan* cartridge are indicated; the peptide that is also present in both MF-4-1 and MF-4-2 is double underlined.

complete 1.7-kb ORF (Fig. 6). Oligonucleotide primers P16 and P18 (Fig. 6) were used to amplify a 2.6-kb fragment from *M. catarrhalis* 035E chromosomal DNA. Nucleotide sequence analysis of this PCR product was used to confirm the nucleotide sequence of the ORF, designated *uspA2*, determined from the ligation-based PCR experiment. When this same PCR product was ligated into the ZAP Express bacteriophage, the resultant recombinant bacteriophage formed plaques which bound MAb 17C7 (data not shown), thus confirming that the *uspA2* ORF encoded a MAb 17C7-reactive protein.

Features of the *uspA2* gene and its encoded protein product. The *uspA2* ORF contained 1,731 nucleotides encoding a protein containing 576 amino acids with a calculated molecular weight of 62,483. Putative −10, −35, and ribosome binding sites are indicated in Fig. 6. Interestingly, there were 15 repeats of the tetranucleotide AGAT located 150 nucleotides 5′ from the predicted translation initiation codon (Fig. 6); the significance of these repeats is not known. An inverted repeat was located immediately downstream from this ORF (nucleotides 2381 to 2412 [Fig. 6]). Immediately upstream from the *uspA2* gene was a gene encoding a product that is most similar to the glycoprotease of *Pasteurella haemolytica* (1). A gene encoding a predicted protein that resembled most closely the MetR regulatory protein of *E. coli* (43) was located downstream from *uspA2* and on the opposite strand. Similar to UspA1, UspA2 contained several different amino acid repeat motifs with two possible leucine zippers (data not shown). When the amino acid sequence of UspA2 was used in a BLAST search of GenBank, the UspA2 protein proved to be most similar (i.e., 27% identical and 47% similar by GAP alignment) to the YadA outer membrane protein of pathogenic *Yersinia* species (59).

The amino acid sequence of UspA2 was 43% identical to that of UspA1. However, closer examination revealed that a region containing amino acids 271 to 411 in UspA2 was 93% identical to the region containing amino acids 498 to 638 in UspA1 (Fig. 7). Outside of these regions, the level of identity was only 22 to 24%. It also should be noted that the four previously described peptides that were not found in UspA1 (Table 2) were found to be very similar to peptides in the deduced amino acid sequence of UspA2. The degree of identity between these four peptides and their counterparts in UspA2 ranged from 67 to 91% (ClustalW score range, 46 to 138). In addition, the peptides which matched or were very similar to peptides in the deduced amino acid sequence of UspA1 (Table 2) also matched peptides found in the deduced amino acid sequence of UspA2.

Construction and analysis of a *uspA2* mutant. To confirm that the 62-kDa UspA2 protein could form the high-molecular-weight UspA antigen, an isogenic *uspA2* mutant was constructed. The oligonucleotide primers P17 and P18 (Fig. 6) were used to amplify a 2-kb product from the *M. catarrhalis* 035E chromosome; this fragment lacked 15 bp from the 5′ end of the *uspA2* ORF. This PCR product was cloned into pBS, yielding the recombinant plasmid pUSPA2. This construct was digested with *Bgl*II, which cut the *uspA2* ORF three times within a 230-bp region (Fig. 6); a *kan* cartridge was inserted into this deletion site, yielding pUSPA2KAN. This mutated DNA was used to electroporate the wild-type strain 035E, and a kanamycin-resistant transformant, designated 035E.2, was selected at random. PCR-based analysis, using oligonucleotide primers P17 and P18 (Fig. 6), indicated that allelic exchange had occurred in strain 035E.2, with the mutated *uspA2* gene replacing the wild-type allele (data not shown).

Western blot analysis with MAb 17C7 determined that outer membrane vesicles of the *uspA2* mutant strain (Fig. 4, lane C) lacked expression of the 85-kDa antigen detected previously in both the wild-type strain and the isogenic *uspA1* mutant (Fig. 4, lanes A and B, respectively). In addition, the very-high-molecular-weight form of the MAb 17C7-reactive antigen was greatly reduced in apparent abundance in outer membrane vesicles from the isogenic *uspA2* mutant (Fig. 4, lane C). The 120-kDa antigen that was missing from the *uspA1* mutant (Fig. 4, lane B) was expressed by the *uspA2* mutant (Fig. 4, lane C).

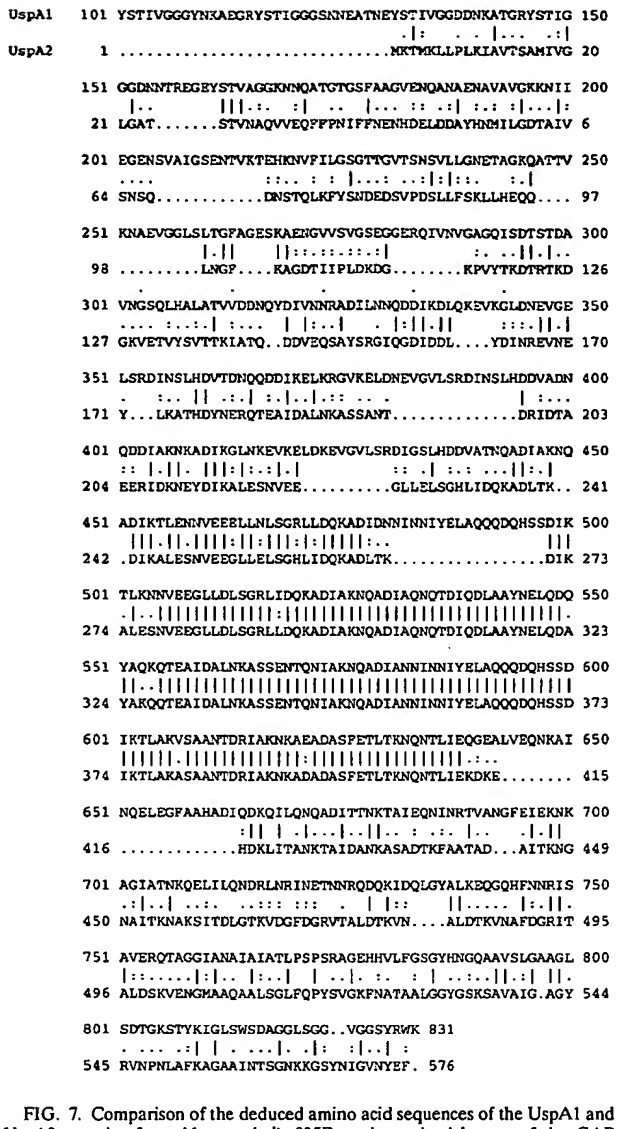


FIG. 7. Comparison of the deduced amino acid sequences of the UspA1 and UspA2 proteins from *M. catarrhalis* 035E as determined by use of the GAP alignment program in the University of Wisconsin sequence analysis package. The first 100 residues of the UspA1 protein are not included. Vertical bars indicate identity, colons indicate conserved substitutions, and single dots indicate less-conserved substitutions.

Southern blot analysis of other *M. catarrhalis* strains by use of *uspA1*- and *uspA2*-specific probes. Chromosomal DNA extracted from *M. catarrhalis* 035E, from four additional disease isolates of this organism, and from *M. catarrhalis* ATCC 25240 was digested with *Pvu*II and used in Southern blot analysis with *uspA1*- and *uspA2*-specific DNA probes. For each strain, the *Pvu*II fragment that bound the *uspA1*-specific probe was dif-

ferent from the *Pvu*II fragment that bound the *uspA2*-specific probe (data not shown).

DISCUSSION

Previous studies from both this laboratory (27) and another (34) described a very-high-molecular-weight protein of *M. catarrhalis* that is expressed by all *M. catarrhalis* disease isolates examined to date. This protein, designated UspA (27) or HMWP (34), has also been shown to be a target for a MAb (17C7) which enhanced pulmonary clearance of this organism in a murine model system and bound to all strains of *M. catarrhalis* tested to date (27). However, the findings of the current study indicate that there are two genes in *M. catarrhalis* 035E that encode the epitope that binds MAb 17C7.

From the available mutant strain analysis data, it is clear that the *uspA1* and *uspA2* gene products can be detected as 120- and 85-kDa proteins, respectively, in Western blot analysis (Fig. 4). These two MAb 17C7-reactive antigens likely represent the monomeric forms of the UspA1 and UspA2 proteins, respectively. At this time, we do not know why these two proteins migrate more slowly in SDS-PAGE (i.e., they have apparent molecular weights of 120,000 and 85,000) than would be expected from their calculated molecular weights (i.e., 88,271 and 62,483). It is interesting to note that the calculated molecular weights for both UspA1 and UspA2 are 73% of the apparent molecular weights of their putative monomeric forms as estimated by SDS-PAGE.

It is also now apparent that both UspA1 and UspA2 form aggregates or oligomers that migrate with an estimated molecular weight of at least 250,000 in SDS-PAGE (Fig. 4). The need to overexpose the autoradiogram to detect the 120- and 85 kDa proteins (Fig. 4) suggested that relatively few of these putative monomeric forms are present in *M. catarrhalis* cells. In addition, it would also appear that the UspA2 protein likely comprises the bulk of the protein present in the very-high-molecular-weight form of the UspA protein in strain 035E (Fig. 4, compare lanes B and C).

The fact that MAb 17C7 binds to the surface of whole *M. catarrhalis* cells (27) indicates that the homologous epitope is exposed to the environment. Whether both UspA1 and UspA2 are exposed on the surface of *M. catarrhalis* cannot be determined from the available data. The deduced amino acid sequence of UspA2 contained what could be a leader peptide, with a hydrophobic region of approximately 20 amino acids at the N terminus that was preceded by one or two basic amino acids (Fig. 6). In contrast, with the assumption that the translation initiation codon depicted in Fig. 2 is correct, the N-terminal region of UspA1 (Fig. 2) did not resemble a leader peptide. At this time, we cannot formally exclude the possibility that the UspA1 protein detected in Western blot analysis of outer membrane vesicles (Fig. 4) is primarily localized elsewhere in the *M. catarrhalis* cell. Conclusive determination of which of these proteins is surface exposed will necessarily have to await development of polyclonal antibody or MAb probes specific for each protein.

The apparent molecular weight of UspA in SDS-PAGE has been reported previously as ranging from 300,000 to 700,000 (27, 34). Treatment of either purified UspA or *M. catarrhalis* outer membrane vesicles with reducing agents, alkylating agents, or heat failed to alter the migration characteristics of this macromolecule in SDS-PAGE (27, 34). However, formic acid treatment of purified, very-high-molecular-weight UspA prior to SDS-PAGE was reported to result in the appearance of a single band with an apparent molecular weight of between 120,000 and 140,000 (34). The 120-kDa antigen expressed by the *uspA1* gene in the present study (Fig. 4) is very similar in size to this product obtained from the formic acid-treated UspA (34), suggesting that UspA1 may have been selectively purified in this previous study. Alternatively, the *M. catarrhalis* strain used in this previous study (34) may have expressed only or mostly UspA1.

The UspA1 protein resembled most closely a surface fibril-forming macromolecule from *H. influenzae* type b that has been implicated in the ability of this encapsulated organism to attach to epithelial cells (60, 61). Genetic analysis of an isogenic *hsf* mutant of *H. influenzae* type b indicated that this mutant lost essentially all of its ability to adhere to Chang conjunctival cells in vitro (60). Similarly, a recombinant *E. coli* strain that expressed the *hsf* gene product exhibited a greatly increased ability to adhere to these same epithelial cells (61). Whether UspA1 can form fibrils on the surface of *M. catarrhalis* or plays some role in the ability of *M. catarrhalis* to colonize the nasopharynx cannot be determined from the available data.

The *M. catarrhalis* UspA2 protein resembled a virulence factor of another pathogen, being most similar to the YadA adhesin-invasin expressed by pathogenic *Yersinia* species. YadA has been implicated in numerous virulence-associated phenotypes, including serum resistance of *Yersinia enterocolitica* (8, 9, 50), adherence to and entry into HEp-2 cells by *Yersinia pseudotuberculosis* (4), binding of *Yersinia* organisms to extracellular matrix components including fibronectin and/or collagen (54, 55, 63, 64), and formation of surface tendrils (fibrillae) (31, 39, 69). Of particular interest, with respect to its similarity to UspA2, is the fact that the native YadA antigen migrates with an apparent molecular weight of approximately 200,000 in SDS-PAGE (58, 69). This 200-kDa antigen actually represents an oligomer comprised of several 45- to 50-kDa YadA monomers (40).

At the level of primary structure, UspA1 and UspA2 are not very similar to each other except in one specific region. There is only 22% identity between UspA1 residues 1 to 450 and UspA2 residues 1 to 240 and just 24% identity between UspA1 residues 649 to 831 and UspA2 residues 415 to 576. In contrast, there is 93% identity between UspA1 residues 498 to 638 and UspA2 residues 271 to 411 (Fig. 7). Furthermore, these two regions both contain the 23-residue peptide that may contain the epitope that binds MAb 17C7 (Fig. 2 and 6), with this peptide being repeated once in UspA1 (Fig. 2). The genetic basis for this apparent duplication of DNA internal to both genes is not known at this time.

The available data indicate that *M. catarrhalis* 035E expressed both UspA1 and UspA2. Southern blot analysis using probes specific for *uspA1* and *uspA2* revealed that other disease isolates of *M. catarrhalis* each possessed two distinct chromosomal DNA fragments that hybridize with these probes (data not shown). This latter result suggests that most disease isolates of this pathogen have the potential to express both UspA1 and UspA2. Determination of whether all strains of *M. catarrhalis* simultaneously express both of these macromolecules will require the development of the UspA1- and UspA2-specific antibody probes mentioned above.

The likelihood that *M. catarrhalis* expresses both of these proteins necessitates reevaluation of published reports concerning the antigenic and immunogenic properties of the UspA protein. Antibodies to UspA have been demonstrated to be present in convalescent-phase sera from patients with documented *M. catarrhalis* pneumonia (27), providing evidence that UspA is expressed by *M. catarrhalis* growing in vivo. It has also been suggested that UspA may play a role in the resistance of some *M. catarrhalis* isolates to killing by normal human

serum (66). Most recently, it was shown that immunization with purified UspA resulted in enhanced pulmonary clearance of homologous and heterologous *M. catarrhalis* strains from the lungs of mice (7). All of these previous studies need to be reinterpreted with respect to the identity of the antigen or immunogen (i.e., UspA1, UspA2, or both) being examined.

The functions of UspA1 and UspA2 in *M. catarrhalis* remain to be defined. It is noteworthy that both UspA1 and UspA2 resemble prokaryotic adhesins, and the possibility that these macromolecules are somehow involved in the adherence of *M. catarrhalis* to human nasopharyngeal mucosa is particularly intriguing. It also has been suggested, in a preliminary report, that the UspA protein may be involved in binding of vitronectin and in complement resistance (66). Direct investigation of the possible involvement of the UspA1 and UspA2 proteins in the interaction between *M. catarrhalis* and components of both the human respiratory tract and the complement system should now be feasible.

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Editor: P. E. Orndorff

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  AUTHORS   Hansen,E.J.
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  TITLE     A protective epitope of Moraxella catarrhalis is encoded by two
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